ORIGINAL ARTICLE

Decreased xanthine oxidoreductase is a predictor of poor prognosis in early-stage gastric cancer

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Background: Xanthine oxidoreductase (XOR) is a key enzyme in the degradation of DNA, RNA and high-energy phosphates. About half of the patients with breast cancer have a decrease in XOR expression. Patients with breast cancer with unfavourable prognosis are independently identified by the loss of XOR.
Aim: To assess the clinical relevance of XOR expression in gastric cancer.
Methods: XOR levels were studied by immunohistochemistry in tissue microarray specimens of 337 patients with gastric cancer and the relation between XOR expression and a series of clinicopathological variables, as well as disease-specific survival, was assessed.
Results: XOR was moderately decreased in 41% and was undetectable in another 14% of the tumours compared with the corresponding normal tissue. Decreased XOR was associated with advanced stage, deep tumour penetration, diffusely spread tumour location, positive lymph node status, large tumour size, non-curative disease, cellular aneuploidy, high S-phase fraction and high cyclooxygenase-2 expression, but not with p53 expression or Borrmann classification. Down regulation of XOR was associated with unfavourable outcome, and the cumulative 5-year gastric cancer-specific survival in patients with strong XOR expression was 47%, compared with 22% in those with moderate to negative expression ($p<0.001$). This was also true in patients with stage I–II ($p = 0.01$) and lymph node-negative ($p = 0.02$) disease, as well as in patients with smaller ($\leqslant 5$ cm) tumours ($p = 0.02$).
Conclusion: XOR expression in gastric cancer may be a new marker for a more aggressive gastric cancer biology, similar to that previously reported for breast cancer.

The major purine compounds in the cell are adenine and guanine ribonucleotides, and deoxyribonucleotides and nucleic acids. They play an essential part in energy-requiring reactions, in nucleic acid synthesis and as signalling molecules. Xanthine oxidoreductase (XOR) catalyses the final reactions of purine catabolism in humans and oxidises hypoxanthine to xanthine and on to uric acid. XOR is coded for by a single gene located on human chromosome 2p22[1] and the protein is mainly expressed in the cytoplasm of hepatocytes, small intestinal enterocytes and goblet cells, vascular endothelial cells and breast epithelium.[2 3] Hypoxia activates XOR both at the transcriptional and post-transcriptional levels,[4 5] and proinflammatory cytokines induce XOR transcription in cell culture.[6 7]

Progressive decrease of XOR activity has been shown in the mouse breast during carcinogenesis,[8] and XOR activity[9] and protein[10] are decreased in rat hepatomas compared with the corresponding normal tissues. Mouse colon carcinomas show considerably decreased XOR activities compared with analogous normal tissue,[11] but to date, no previous reports describing the distribution of XOR in normal and malignant gastric epithelium in rodents or humans have been published. By using tissue microarray samples from a large population-based cohort of breast cancer, with well-characterised clinicopathological parameters and outcome data, we recently showed that XOR is down regulated in more than half of the breast tumours, and that absence of XOR is an independent predictor of unfavourable outcome.[12]

Given that XOR is down regulated in breast cancer,[12 13] and as XOR is strongly expressed in the epithelial cells of the proximal intestine,[2] we hypothesised that the protein may also be differentially expressed in patients with gastric cancer. To deal with this question, we examined the expression of XOR in a consecutive series of surgically treated patients with gastric cancer and analysed whether the expression of XOR is associated with clinicopathological parameters and clinical outcome.

MATERIALS AND METHODS

Patients

The study included 337 consecutive patients who underwent surgery for histologically verified gastric adenocarcinoma at the Helsinki University Central Hospital from 1983 to 1996. Tissue specimens suitable for immunohistochemical evaluation of XOR expression were available in 264 patients. Staging was performed according to the Union Internationale Contre le Cancer classification of 1992.[14] Survival data were available for all patients and obtained from patient records, the Finnish Cancer Registry and the Population Registry in Finland. The median follow-up time for patients who were alive at the end of follow-up was 12.5 (range 4.7–20.8) years. The clinicopathological characteristics of the patients in this series have been previously reported.[15]

Preparation of tumour tissue microarrays

Representative tumour regions in routinely fixed paraffin-wax-embedded samples were first defined from haematoxylin and eosin-stained sections and marked. Blocks of paraffin-wax-embedded donor tissue were sampled with 0.6-mm punchers by using a tissue microarray instrument (Manuel Tissue Arrayer 1, Beecher Instruments, Silver Spring, Maryland, USA). Three cores were cut from each donor block for the tissue microarray blocks. From the

Abbreviations: COX-2, cyclooxygenase-2; SPF, S-phase fraction; XOR, xanthine oxidoreductase

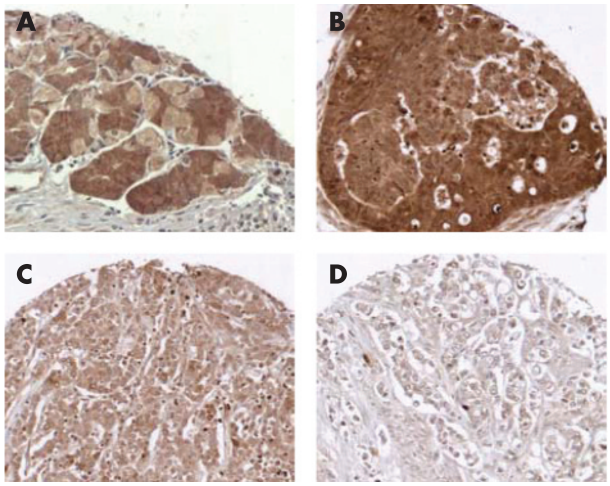

Figure 1 Immunohistochemical staining of xanthine oxidoreductase in normal gastric epithelium (A) and in gastric adenocarcinoma (B–D) as described in Materials and methods. Examples of strong (B) moderate (C) and no (negative) (D) immunoreactivity in the cytoplasm of tumour cells.

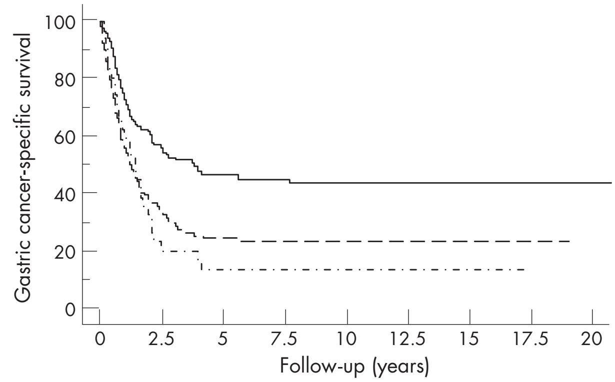

Figure 2 Disease-specific survival of 264 patients with gastric cancer according to xanthine oxidoreductase expression. ······, strong (n = 118); ---, moderate (n = 110); —, negative (n = 36). $\chi^2 = 17.0$; $p \leqslant 0.001$ (log rank test for trend).

tumour samples available, six tissue array blocks were prepared, each containing 80–180 tumour samples.[16] Sections of 4 μm thickness were cut and processed for immunohistochemistry.

Immunohistochemistry

The antigen was enhanced in Target Retrieval Solution, pH 6.0 (DAKO, Carpentaria, California, USA), at 95–97℃ for 30 min on routinely processed paraffin-wax sections. The sections were then treated with 3% hydrogen peroxide and XOR protein was detected using a well-characterised rabbit polyclonal anti-XOR antibody.[2 17] The antibody was diluted 1:50 in Blocking Solution (Powervision, Immunovision, Daly City, California, USA), and incubated with the samples (overnight at 4℃). An antimouse-peroxidase polymer (30 min at room temperature) with diaminobenzidine as a chromogen (Powervision) was used for visualisation. Specificity of the XOR localisation was confirmed by staining slides with pre-immune serum and without the primary antibodies.

Immunostaining for DNA ploidy, S-phase fraction (SPF),[18] cyclooxygenase (COX-2)[19] and p53[20] was carried out using established procedures.

Digitisation of stained tissue microarray slides

The tissue microarray slides immunostained for XOR were digitised at 0.5 μm resolution and made available for viewing on our website (http://www.webmicroscope.net/supplements/xor). For image acquisition, we used a Zeiss Axioskop2 MOT microscope (Zeiss Gmbh, Göttingen, Germany) equipped with a NeoFluar oil ×40 objective, Märzhauser motorised specimen stage (Märzhauser, Wetzlar, Germany) and a charge-coupled device camera (Zeiss Axiocam HR, Carl Zeiss Medtec, Dublin, California, USA). Image acquisition was controlled by the KS400 software (Zeiss). The acquired image files were digitally sharpened and stitched into a single montage file, which was compressed into a wavelet-type image file (enhanced compressed wavelet format) with the ER Mapper software (Earth Resource Mapping Pty, West Perth, Australia). The compressed virtual slides were uploaded to the web server running the Image Web Server software (Earth Resource Mapping Pty). The virtual slides on the website can be viewed at any magnification level on a standard web browser (MS Internet Explorer or Netscape on any Windows platform).

Scoring of XOR immunostaining

Expression of XOR was evaluated from the digitised slides described above by one of the investigators (NL). The investigator was blinded to the clinicopathological data at the time of scoring. Cytoplasmic XOR staining intensity was scored as follows: strong, staining comparable with that of the normal gastric epithelial cells; moderate, clearly decreased staining; and negative, no staining for the XOR protein in >90% of the cancer cells.

Statistical analysis

The associations between XOR expression and other clinicopathological factors were analysed with the χ^2 test or Fisher's exact test in the case of low expected frequencies. Life tables were calculated according to the Kaplan–Meier method. Deaths from gastric cancer were included, whereas those from other causes were excluded. Survival curves were compared with the log rank test or the log rank test for trend in the case of three or more ordered categories. Multivariate survival analyses were performed with the Cox proportional hazards model, using a backward stepwise selection of variables, and a significance level of 0.05 was adopted as the limit for including a covariate. The assumption of proportional hazards was ascertained with complementary log plots. All statistical tests are two sided. The statistical software used was SPSS V.13.0.

RESULTS

XOR expression in normal gastric epithelium and in gastric cancer

XOR was strongly expressed in surface epithelial cells, whereas the parietal cells of the normal gastric mucosa showed moderate immunoreactivity.

Cytoplasmic XOR was scored into three categories in 264 specimens with gastric cancer. More than half the gastric carcinomas had a lower XOR level in the cytoplasm than the corresponding normal epithelium. Of the tumours, 45% (n = 118) showed strong staining for cytoplasmic XOR, similar to the XOR expression in the normal gastric epithelial cells, whereas 41% (n = 110) showed moderate staining, corresponding to a decreased XOR expression, and 14% (n = 36) had no cytoplasmic XOR expression (fig 1). The cytoplasm of the stromal cells scored negative or weakly positive for the XOR protein and the nuclei were moderately stained.

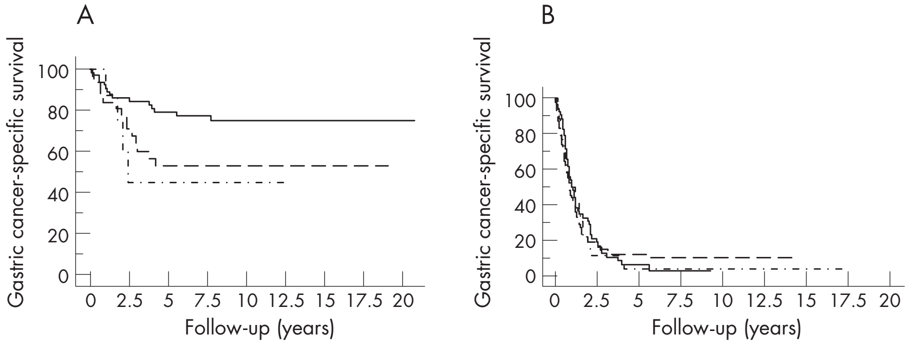

Figure 3 (A) Disease-specific survival of 106 patients with gastric cancer with stage I–II disease according to xanthine oxidoreductase protein expression. ·····, strong (n = 65); ---, moderate (n = 31); —, negative (n = 10). $\chi^2 = 6.00$; p = 0.01 (log rank test for trend). (B) Disease-specific survival of 158 patients with gastric cancer with stage III–IV disease according to XOR protein expression. ·····, strong (n = 53); ---, moderate (n = 79); —, negative (n = 26). $\chi^2 = 0.16$; p = 0.69 (log rank test for trend).

The heterogeneity of XOR expression was minimal in 15 slides of whole tumour sections that were studied. Therefore, the staining results obtained from the tissue microarray cores were considered to be representative of the entire tumour.

Association between cytoplasmic XOR expression and clinicopathological parameters

Decreased cytoplasmic XOR expression was markedly associated with advanced stage, deep tumour penetration, diffusely spread tumour location, positive lymph node status, large tumour size, non-curative disease, cellular aneuploidy, high SPF and high COX-2 score. No significant association was found between cytoplasmic XOR and sex, Borrmann classification or p53 expression (table 1).

Association of XOR expression with gastric cancer-specific survival

Decreased XOR expression (moderate and negative) compared with the normal gastric epithelium was markedly associated with decreased cancer-specific survival among the 264 patients with gastric cancer (fig 2). The 5-year gastric cancer-specific survival in patients with strong XOR expression was 47%, compared with 24% in those with moderate staining (hazards ratio 1.86; p = 0.002) and 13% in those

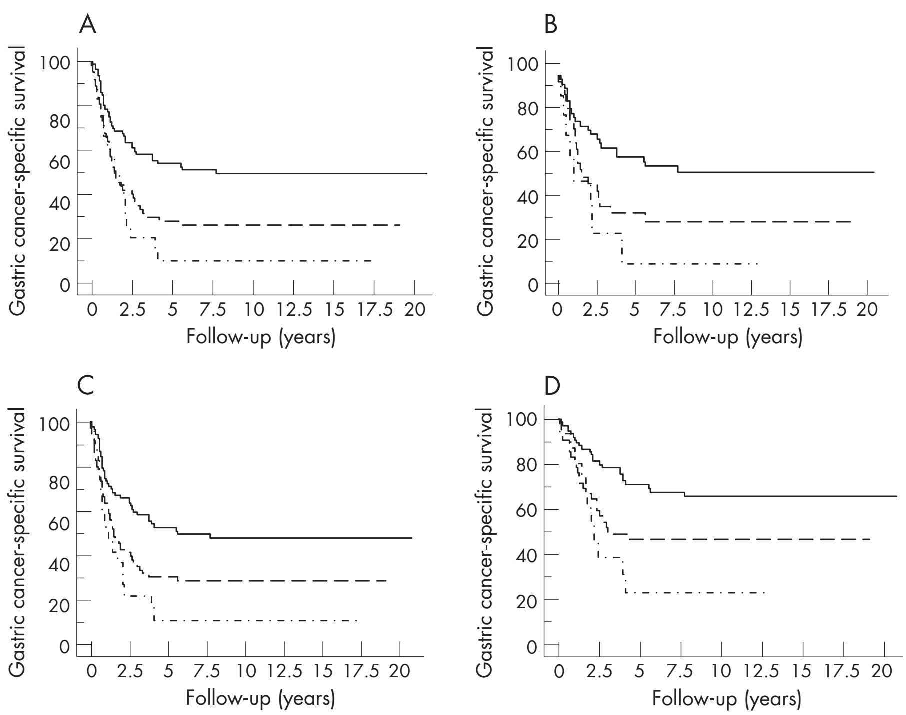

Figure 4 (A) Disease-specific survival of 168 patients with gastric cancer with diploid tumour cells according to xanthine oxidoreductase (XOR) protein expression. ·····, strong (n = 81); ---, moderate (n = 63); —, negative (n = 24). $\chi^2 = 15.7$; $p < 0.001$ (log rank test for trend). (B) Disease-specific survival of 98 patients with gastric cancer with a low S-phase fraction (SPF<7.6) in tumour cells according to XOR protein expression. ·····, strong (n = 53); ---, moderate (n = 34); —, negative (n = 11). $\chi^2 = 9.90$; p = 0.002 (log rank test for trend). (C) Disease-specific survival of 175 patients with gastric cancer with a low level of p53 ($\leqslant$20%) in tumours cells according to XOR protein expression. ·····, strong (n = 83); ---, moderate (n = 68); —, negative (n = 24). $\chi^2 = 14.8$; p = 0.001 (log rank test for trend). (D) Disease-specific survival of 136 patients with gastric cancer operated with a curative intent according to XOR protein expression. ·····, strong (n = 76) ---, moderate (n = 43); —, negative (n = 17). $\chi^2 = 13.2$; p = 0.003 (log rank test for trend).

Table 1 Distribution of xanthine oxidoreductase immunoreactivity according to clinicopathological characteristics in 264 patients with gastric cancer

Clinicopathological variable	n	XOR Strong n (%)	Moderate n (%)	Negative n (%)	χ^2	p Value
Age at diagnosis (years)						
<67	120	64 (53)	37 (31)	19 (16)	10.65	0.005
⩾67	144	54 (37)	73 (51)	17 (12)		
Sex						
Female	142	62 (44)	64 (45)	16 (11)	2.19	0.334
Male	122	56 (46)	46 (37)	20 (17)		
Tumour location						
Upper third	57	21 (37)	32 (56)	4 (7)	20.13	0.01
Middle third	88	42 (48)	31 (35)	15 (17)		
Lower third	100	50 (50)	38 (38)	12 (12)		
Diffusely spread	12	1 (8)	6 (50)	5 (42)		
Stump	5	2 (40)	3 (60)	0		
TNM stage						
IA–IB	75	53 (70)	17 (23)	5 (7)	31.1	⩽0.001
II	31	12 (39)	14 (45)	5 (16)		
IIIA–IIIB	80	29 (36)	41 (51)	10 (13)		
IV	78	24 (31)	38 (49)	16 (20)		
Penetration depth						
Organ-defined mucosa—subserosa	87	60 (69)	21 (24)	6 (7)	31.0	⩽0.001
Serosa—adjacent structures	177	58 (33)	89 (50)	30 (17)		
Lymph node metastases						
N0	117	70 (60)	37 (32)	10 (8)	20.74	0.004
N1	77	28 (36)	36 (47)	13 (17)		
N2	70	20 (29)	37 (53)	13 (18)		
Distant metastasis						
No	191	95 (50)	74 (39)	22 (11)	7.6	0.022
Yes	73	23 (32)	36 (49)	14 (19)		
Tumour size (cm)						
⩽5	146	81 (56)	53 (36)	12 (8)	19.93	0.001
>5	113	33 (29)	57 (50)	23 (21)		
Borrmann classification						
Type I	47	18 (38)	26 (55)	3 (7)	9.3	0.16
Type II	77	39 (51)	26 (34)	12 (15)		
Type III	63	24 (38)	26 (41)	13 (21)		
Type IV	33	14 (42)	12 (37)	7 (21)		
Laurén classification						
Intestinal	129	65 (50)	50 (39)	14 (11)	3.77	0.15
Diffuse	135	53 (40)	60 (44)	22 (16)		
Grade of differentiation						
1	15	7 (47)	6 (40)	2 (13)	2.05	0.92
2	42	18 (43)	21 (50)	3 (7)		
3	55	22 (40)	27 (49)	6 (11)		
4	4	1 (25)	2 (50)	1 (25)		
Curativity						
Intent to cure	136	76 (56)	43 (32)	17 (12)	11.84	0.003
Non-curative	113	39 (35)	57 (50)	17 (15)		
DNA ploidy						
Diploid	168	81 (48)	63 (38)	24 (14)	7.46	0.024
Aneuploid	75	24 (32)	42 (56)	9 (12)		
SPF						
Low <7.6%	98	53 (54)	34 (35)	11 (11)	6.89	0.031
High ⩾7.6%	121	44 (36)	58 (48)	19 (16)		
p53						
⩽20%	175	83 (47)	68 (39)	24 (14)	1.85	0.4
>20%	89	35 (39)	42 (47)	12 (14)		
COX-2						
0–1	104	54 (52)	35 (34)	15 (14)	5.01	0.008
2–3	154	61 (40)	73 (47)	20 (13)		

COX-2, cyclooxygenase-2; SPF, S-phase fraction; TNM, tumour, nodes and metastasis; XOR, xanthine oxidoreductase.

with negative staining (hazards ratio 2.14; $p = 0.002$) of the cytoplasm (table 2). For further survival analysis, patients with strong XOR expression were compared with the combined group of patients with moderate and negative XOR staining. Patients with strong XOR expression had a 5-year survival of 47%, whereas those with moderate or no XOR expression had a disease-specific survival of only 22% ($p<0.001$; table 2).

Decreased XOR expression was markedly associated with poor outcome in patients with stage I–II disease, negative lymph node status, tumour size <5 cm, diploid tumours, low and high SPF counts, low and high p53 and low COX-2 score, and in patients operated with a curative intent (table 2, figs 3 and 4).

Multivariate analysis

In multivariate analysis, T (tumour), N (node) and M (metastasis) were the strongest independent prognostic factors, followed by COX-2. XOR and age at diagnosis did not predict survival independently (table 3).

Table 2 Five-year gastric cancer-specific survival according to cytoplasmic xanthine oxidoreductase expression

Clinicopathological variable	XOR score	n	5-year gastric cancer-specific survival, % (95% CI)	χ^2	p Value	HR (95% CI)
XOR expression	Strong	118	47 (37 to 56)	Ref	Ref	1
	Moderate	110	24 (15 to 33)	13.68	0.002	1.86
	Negative	36	13 (0 to 27)	11.39	0.001	2.14
	Strong	118	47 (37 to 56)	17.83	<0.001	1.92
	Moderate–negative	146	22 (14 to 29)			
Stage						
I–II	Strong	65	79 (68 to 90)	6.25	0.012	2.34
	Moderate–negative	41	52 (35 to 69)			
III–IV	Strong	53	6 (0 to 13)	0.12	0.73	1.06
	Moderate–negative	105	10 (3 to 16)			
Lymph node status						
Negative	Strong	70	73 (62 to 85)	5.62	0.018	2.05
	Moderate–negative	47	48 (32 to 65)			
Positive	Strong	48	7 (0 to 15)	0.13	0.72	1.07
	Moderate–negative	99	10 (3 to 16)			
T status						
1–2	Strong	60	79 (68 to 90)	1.12	0.29	1.57
	Moderate–negative	27	63 (43 to 84)			
3–4	Strong	58	13 (3 to 23)	0.48	0.49	1.13
	Moderate–negative	119	12 (5 to 18)			
Tumour size (cm)						
$\leqslant 5$	Strong	81	58	9.52	0.002	2.01
	Moderate–negative	65	35			
>5	Strong	33	21 (5 to 37)	0.17	0.68	1.1
	Moderate–negative	80	12 (4 to 20)			
Intent to cure						
Curative	Strong	76	71 (60 to 82)	10.6	0.001	2.33
	Moderate–negative	60	41 (27 to 54)			
Non-curative	Strong	39	3 (0 to 8)	0.51	0.48	1.16
	Moderate–negative	74	3 (0 to 7)			
DNA ploidy						
Diploid	Strong	81	54 (42 to 66)	14.2	0.002	2.11
	Moderate–negative	87	23 (14 to 33)			
Aneuploid	Strong	24	13 (−1 to 26)	0.31	0.58	1.16
	Moderate–negative	51	16 (5 to 27)			
SPF						
Low <7.6%	Strong	53	63 (49 to 76)	8.07	0.005	2.19
	Moderate–negative	45	32 (16 to 48)			
High $\geqslant$7.6%	Strong	44	26 (12 to 41)	4.78	0.029	1.59
	Moderate–negative	77	13 (5 to 20)			
p53						
Low	Strong	83	52 (41 to 64)	12.4	0.004	1.98
	Moderate–negative	92	26 (16 to 35)			
High	Strong	35	33 (17 to 49)	4.52	0.033	1.72
	Moderate–negative	54	14 (3 to 26)			
COX-2						
0–1	Strong	54	73 (60 to 86)	14.2	0.002	2.99
	Moderate–negative	50	33 (19 to 47)			
2–3	Strong	61	21 (10 to 32)	3.31	0.069	1.4
	Moderate–negative	93	14 (5 to 22)			
Laurén classification						
Intestinal type	Strong	65	40 (27 to 53)	7.74	0.005	1.82
	Moderate–negative	64	21 (9 to 33)			
Diffuse type	Strong	53	55 (40 to 69)	11	0.001	2.13
	Moderate–negative	82	22 (13 to 32)			

COX-2, cyclooxygenase-2; SPF, S-phase fraction; XOR, xanthine oxidoreductase.

DISCUSSION

In this study, we examined the expression of XOR in a series of patients with gastric cancer to elucidate the association between XOR and clinicopathological parameters as well as disease-specific survival. XOR was recently shown to be decreased or undetectable in more than half of the tumours in a large series of studies on breast cancer and was independently associated with survival.[12] Although XOR is down regulated in virtually all rodent and human cancers studied,[10 21 22] the report by Linder et al[12] is the only study that provides information about the association between XOR and cancer outcome. In this study, down regulation of XOR is shown to be common in patients with gastric cancer and associated with unfavourable disease-specific survival.

Decreased cytoplasmic expression of XOR was associated with several adverse prognostic features such as high tumour, nodes and metastasis stage, deep penetration, lymph node metastases, large size, non-curative and diffusely spread disease. Decreased XOR was also seen more often in tumours of the aneuploid type, a high S-phase fraction and strong COX-2 expression, but was not markedly associated with p53, Borrmann type or Laurén classification. In breast cancer, decreased XOR expression was also associated with several unfavourable features such as high histological grade, large size, large number of positive axillary lymph nodes and high COX-2 expression, although the associations were relatively weak. According to the results of this study, XOR seems to exhibit a stronger association with markers of disease progression in patients with gastric cancer than in those with breast cancer.

Univariate survival analyses, carried out to evaluate the prognostic value of the XOR levels, showed that patients whose tumour tissue contained less immunoreactive XOR than normal epithelial cells had about twice the risk of dying

Table 3 Multivariate survival analysis: Cox proportional hazards regression models for distant disease-free survival in 243 patients with gastric cancer

Clinicopathological variable	β-coef	p Value	χ^2	HR	95% CI
T1				1.00	
T2	1.001	0.02	5.25	2.72	1.16 to 6.40
T3	1.688	<0.001	16.51	5.41	2.40 to 12.22
T4	2.217	<0.001	26.32	9.18	3.93 to 21.40
N0				1.00	
N1	0.655	0.001	10.29	1.93	1.29 to 2.87
N2	1.140	<0.001	26.66	3.13	2.03 to 4.82
M (M0 v M1)	0.667	<0.001	16.16	1.95	1.41 to 2.70
COX-2 expression (low v high)	0.434	0.005	7.90	1.54	1.14 to 2.09
XOR (strong v moderate and negative)		NS			
Age at diagnosis (<50; 50–69; ⩾70 years)		NS			

β-coef, regression coefficient; COX-2, cyclooxygenase-2; M, metastasis; N, node; NS, not significant; T, tumour; XOR, xanthine oxidoreductase.
Age at diagnosis was entered as a continuous variable. T status, number of positive lymph nodes and XOR expression were entered as categorical variables, and tumour size and the occurrence of distant metastasis as binary variables.

from gastric cancer as those whose cancer strongly expressed XOR. In a subgroup analysis in this study, decreased XOR was associated with a worse outcome in patients who generally have a better prognosis, such as those with stage I–II disease, smaller tumours, node-negative disease, diploid tumours and tumours with a low SPF fraction and low p53. Our previous study on patients with breast cancer showed similar results—that is, a low XOR level of carcinomas was associated with unfavourable survival. Patients with breast cancer with no XOR expression had more than twice the risk of distant recurrence as those with a moderately decreased or normal expression. This was also true in patients with node-negative breast cancer and in patients with small (⩽1 cm) tumours.

Decreased XOR was not associated with a worse prognosis in an advanced stage of gastric cancer. This could be the reason for XOR not being retained as an independent prognostic marker in multivariate survival analysis.

In future, it will be important to elucidate whether the lowered XOR expression in about half of the tumours is caused by silencing of the XOR gene or by post-translational modifications of the protein and whether these modifications are only indirectly associated with the pathogenesis of cancer. Despite the evidence of a role for XOR in cancer, the enzyme has previously been studied in only a limited number of human tumours.[13 23] Although the (patho)physiological role of XOR is still controversial, it has become clear only in the past years that the function of this protein is not limited to its role in purine degradation. Mice heterozygotic for a loss of function in the XOR gene (XOR+/−) are unable to maintain lactation because of membrane defects in the milk fat droplets and disruption of the mammary epithelial cells,[3] indicating that XOR may have a structural role in the development of the mammary gland, which may also apply to the gastric epithelium.

In conclusion, about half the gastric carcinomas studied were characterised by a decreased XOR antigen level compared with the corresponding normal tissue, similar to that previously shown for breast cancer. Although the underlying biological mechanisms that down regulate XOR cancer in general, as in gastric cancer, have not been seen, it seems to be clinically relevant for the survival of patients, in agreement with our previous results for breast cancer.

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